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Chitin Degradation Proteins Produced by the Marine Bacterium *Vibrio harveyi* Growing on Different Forms of Chitin

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Relatively little is known about the number, diversity, and function of chitinases produced by bacteria, even though chitin is one of the most abundant polymers in nature. Because of the importance of chitin, especially in marine environments, we examined chitin-degrading proteins in the marine bacterium *Vibrio harveyi*. This bacterium had a higher growth rate and more chitinase activity when grown on β-chitin (isolated from squid pen) than on α-chitin (isolated from snow crab), probably because of the more open structure of β-chitin. When exposed to different types of chitin, *V. harveyi* excreted several chitin-degrading proteins into the culture media. Some chitinases were present with all of the tested chitins, while others were unique to a particular chitin. We cloned and identified six separate chitinase genes from *V. harveyi*. These chitinases appear to be unique based on DNA restriction patterns, immunological data, and enzyme activity. This marine bacterium and probably others appear to synthesize separate chitinases for efficient utilization of different forms of chitin and chitin by-products.

Chitin is one of the most abundant biopolymers in nature and possibly the most abundant in the marine environment, since it is produced by many marine organisms, including zooplankton and several phytoplankton species (3). Chitin consists of β-1,4-linked *N*-acetylglucosamine residues that are arranged in antiparallel (α), parallel (β), or mixed (γ) strands, with the α configuration being the most abundant. The degree of deacetylation also varies from 0 to 100% (chitosan). Except for the β-chitin of diatoms (chitan), chitin is always found cross-linked to other structural components, such as proteins and glucans (3).

Given the great diversity of possible chitin structures, perhaps it is not surprising that bacteria typically produce more than one type of chitinase. *Bacillus circulans* WL-12 secretes at least six major chitinases which differ in enzyme activity (24). Five unique chitinolytic enzymes have been isolated from *Serratia marcescens*, although not all of these have been cloned (2, 5, 8). Three separate chitinase genes have been identified in *Streptomyces lividans* (9). Chitinases with diverse enzymatic properties may be synthesized from separate genes, as in *Streptomyces lividans*, or result from proteolytic processing, as occurs with a chitinase in an *Alteromonas* sp. (23) and *Streptomyces olivaceoviridis* (17). While it is known that bacteria can produce more than one type of chitinase, no attempt has been made to identify all the chitinase genes in a single organism, with the possible exception of *B. circulans* WL-12.

Presumably, a bacterium produces different chitinases to efficiently hydrolyze the different forms of chitin found in nature, although this has not been examined. It is known that a single chitinase is not equally efficient hydrolyzing α and β forms of chitin. Shigemasa et al. (20) found that a chitinase isolated from *Bacillus* sp. strain P1-7S degraded β-chitin more efficiently than α-chitin, perhaps because it is more difficult for chitinases to gain access and hydrolyze the tightly packed, antiparallel strands of α-chitin. This may also explain why most organisms have α- rather than β-chitin in exoskeletons or cell walls (3). The hydrolysis of these two forms of chitin by bacteria has not been examined.

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We have been working with *Vibrio harveyi* (11, 12) because previous work has already identified two enzymes involved in chitin degradation by this marine bacterium. The first gene cloned and sequenced encodes a chitiobiase, which cleaves the bond joining the two *N*-acetylglucosamine units in chitobiose (7). The other gene cloned, *chiA*, encodes a chitinase and was tentatively identified as the main chitinase of *V. harveyi* (21). In a related marine bacterium, *Vibrio furnissii*, two other enzymes (chitodextrinase and *N*-acetyl-β-glucosaminidase) involved in the complete degradation of chitin have been examined (1).

One purpose of this study was to begin to identify all chitinase genes and chitinases excreted by *V. harveyi*. We found six unique genes encoding chitin-degrading enzymes and excretion of at least 10 different chitinolytic enzymes depending on the type of chitin the bacteria were grown on. As suggested by previous work with an isolated chitinase (20), we hypothesized that β-chitin would elicit more chitinase activity and would be degraded faster than α-chitin. Our experiments support this hypothesis. This bacterium appears to have a complex system of enzymes and substrate-binding proteins to degrade this abundant biopolymer.

MATERIALS AND METHODS

Strains and vectors. *V. harveyi* BB7 (obtained from Robert Belas and maintained as glycerol stock) was grown in LM (1% tryptone, 0.5% yeast extract, 2% NaCl) or minimal medium (artificial seawater [ASW] supplemented with 0.5% tryptone). The ASW used here contains the nine most common salts found in seawater (11). Synthesis of chitinases was induced by addition of squid pen, snow crab, colloidal (prepared from snow crab chitin by the procedure of Roberts and Cabib [15]), regenerated (prepared by the procedure of Molano et al. [10] with chitosan made from crab), or glycol (prepared from glycol chitosan by the procedure of Trudel and Asselin [22]) chitin. The phagemid pBluescript KS$^-$ (Stratagene) was used for subcloning. All compounds and reagents were purchased from Sigma unless noted otherwise.

Growth and chitinase activity. *V. harveyi* BB7 was grown in ASW containing 2 mM potassium phosphate buffer (pH 7.5) and 10 mg of either snow crab or squid pen chitin per ml (initial cell concentration, 4×10^5 cells/ml). Over time, cell abundance was determined by direct counts with epifluorescent microscopy of acridine orange-stained cells. Aliquots were also removed and assayed for chitinase activity by monitoring fluorescence (with a Hoefer TKO 100 fluorometer) produced during hydrolysis of the chitin analog, 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobioside (MUF-diNAG; final concentration, 50 μM), by cells diluted in ASW over 10 to 30 min. The reaction was stopped, and fluorescence was

enhanced by the addition of a glycine and ammonium hydroxide solution (final concentrations, 5 and 20 mM, respectively).

To examine chitinases excreted in response to different chitins, *V. harveyi* BB7 was grown for 3 days in minimal medium containing 1 mg of squid, snow crab, colloidal, regenerated, or glycol chitin per ml. The cells were pelleted, and the supernatants, containing the excreted proteins, were concentrated with Centriplus concentrators (Amicon). The proportion, number, and size of the various chitinases, excreted after induction with the different chitins, were examined by glycol chitin-sodium dodesyl sulfate-polyacrylamide gel electrophoresis (glycol-chitin-SDS-PAGE), essentially by the procedure of Trudel and Asselin (22). Briefly, 0.01% glycol chitin was added directly to the separating gel. Following electrophoresis, the gel was incubated at 37°C for 2 h in 100 mM sodium acetate buffer (pH 5), containing 1% Triton X-100. The gel was then stained for 5 min in fresh 0.01% Calcofluor white M2R in 500 mM Tris-Cl (pH 8.9) and destained for 1 h in water. Chitinase activity was detected, with UV light, by clearing zones in the gel. Equal amounts of protein (0.4 μg), determined by the *dot*METRIC protein assay (Geno Technology), were added to the SDS-PAGE loading buffer; they were not heated prior to electrophoresis.

Genomic library construction and screening. To isolate chitinase genes from *V. harveyi*, two genomic libraries were constructed. For the plasmid library, genomic DNA was partially digested with *Hin*dIII (New England Biolabs) and loaded onto an agarose gel, and DNA in the size range of 5 to 15 kb was isolated after agarose gel electrophoresis. DNA was purified from gels with Geneclean (Bio 101). Isolated DNA was ligated into pBluescript II KS$^-$ (Stratagene) and then introduced into XL1-Blue MRF′ by electroporation. Colonies were screened for the presence of protein recognized by an antichitinase antibody (11) by the procedure of Sedgwick et al. (19). Briefly, the colonies were lifted onto a dry nitrocellulose circle, sandwiched with another nitrocellulose circle, lysed with 5% SDS, electrophoresed onto the membrane, and then immunoprobed. The clones recognized by antichitinase were then detected by probing with anti-rabbit goat immunoglobulin G conjugated with alkaline phosphatase (Boehringer Mannheim). Enzyme activity was monitored with color development.

A second genomic library was constructed in the phagemid λZapII (Stratagene) to help ensure that all chitinase genes were identified. Genomic DNA was partially digested with *Eco*RI (Boehringer Mannheim) and loaded onto an agarose gel, and DNA in the size range of 5 to 10 kb was isolated. Size-selected DNA was ligated into *Eco*RI-digested λZapII, packaged, allowed to infect cells, and plated as described in the recommended procedure. Plaques were screened for chitinase activity with MUF-diNAG, which when hydrolyzed fluoresces blue after UV excitation (300 nm). Positive plaques were cored, and the plasmid was excised from the phage as described in the manufacturer's instructions. Phages in plaques exposed to this screening method are still able to infect *Escherichia coli* and be amplified. The plasmids obtained were tested to verify the positive results. Activity in plaques or colonies on agar plates was detected by spraying or spotting 50 μM MUF-diNAG (5 mM stock in *N*,*N*-dimethylformamide [DMF]) in 0.1 M phosphate buffer (pH 7.5). The plates were incubated at 37°C for 10 to 30 min and viewed under UV light.

To help determine the type of chitinase activity, clones able to hydrolyze MUF-diNAG were also tested with 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MUF-NAG) and 4-methylumbelliferyl β-D-*N*,*N*′,*N*′′-triacetylchitotriose (MUF-triNAG). Colonies were spotted with 2.5 μl of either MUF-NAG or MUF-triNAG (5 mM stock in DMF), incubated in the dark for 15 to 30 min, and then viewed under UV light.

Construction of pChiA1. Three separate attempts to obtain a clone with a complete *chiA* (the main chitinase of *V. harveyi*) were unsuccessful, and so we cloned a truncated version of *chiA* produced by PCR. ChiA′ was amplified from *V. harveyi* BB7 genomic DNA with primers for the coding region of *chiA′* (5′ATGTTAAAACGTAAAGCTCTACAA and 5′CTGCAGACCAGACACG CTCAGTTC; synthesized by Genosys), which were based on the sequence data from Soto-Gil (21). The single PCR product was 1.7 kb, the same length as that predicted from the sequence information. The resulting amplified *chiA′* was made blunt-ended with mung bean nuclease and ligated into blunt-ended, alkaline phosphatase-treated pBluescript II KS$^-$. After electroporating the ligation reaction into XL1-Blue MRF′, colonies were screened for chitinase activity with MUF-diNAG.

Chitinase activity. To investigate enzymatic properties of the cloned chitinases, we examined hydrolysis of different chitins and chitin analogs by *E. coli* bearing these genes. Cells were pelleted by centrifugation, washed three times with Tris-buffered saline (20 mM Tris, 150 mM NaCl [pH 7.5]), resuspended in Tris-buffered saline, and sonicated (11). Sarcosyl (1% final concentration) was added to the lysed cells, which were then incubated for 1 h at 37°C. The extracts were then centrifuged for 10 min at 9,000 × *g* and the supernatants were used for further experiments.

Chitinase activity in crude extracts was determined with different forms of chitin. ^{3}H-chitin was prepared by growing SAP 93 (an unidentified fungus isolated from Sapelo Island salt marsh by Steve Newell) 1 week in medium containing *N*-acetyl-D-[1-^{3}H]glucosamine (Amersham). The method of Roff et al. (16) was used to isolate the labeled chitin. Briefly, the fungus was harvested, subjected to two cycles of freezing and thawing, and digested with proteinase K. The mixture was then extracted in 5% SDS at 50°C for 30 min. The chitin pellet was rinsed three times with water and then with a methanol-chloroform (1:1) solution. The washed chitin was sonicated to break up the pellet and dried before use. Cell extracts were added to labeled chitin in 10 mM Tris-Cl (pH 7.5) and allowed to incubate overnight at room temperature with gentle shaking. Reactions were filtered onto Whatman GF/F filters, and both filter and filtrate were radioassayed in Ultima Gold scintillation cocktail (Packard) to determine degradation.

Chitinase activity was also examined with chitin analogs. The extracts were added to 100 μl (total volume) of 10 mM Tris-Cl (pH 7.5), containing 50 μM MUF-diNAG, and incubated for 30 min at 37°C. Fluorescence resulting from hydrolysis of MUF-diNAG was measured as described above. The extracts were also tested for activity with another chitin analog, *p*-nitrophenyl-β-D-*N*,*N*′-diacetylchitobioside (NP-diNAG). Extracts were added to 150 μl (total volume) of 10 mM Tris-Cl (pH 7.5), containing 666 μM NP-diNAG (10 mM stock in DMF), and incubated for 30 min at 37°C. The reactions were stopped by the addition of 450 μl of 1 M Tris base, and activity was determined by measuring the A_{410} of released *p*-nitrophenol.

Two types of zymograms were used to attempt to determine the molecular weights of the cloned chitinases. In both procedures, protein extracts were added to the SDS-PAGE loading buffer but were not incubated at 95°C prior to electrophoresis. The first zymogram, glycol chitin-SDS-PAGE, was performed as described above. In the second, chitinase activity was detected by use of the chitin analog MUF-diNAG. Following electrophoresis, the polyacrylamide gel was incubated for 30 min in Triton-acetate buffer (100 mM sodium acetate [pH 5], with 1% Triton X-100) and then transferred to an agarose plate (0.5% agarose, 25 mM Tris, 15 mM NaCl [pH 7.5]) containing MUF-diNAG (10 μM). After about 20 min, the gels were observed with UV light. The protein bands with chitinase activity fluoresce blue.

RESULTS

Chitinase activity in *V. harveyi*. To examine the degradation and response of marine bacteria to chitin, *V. harveyi* was grown on either α-chitin (snow crab) or β-chitin (squid pen) (Fig. 1). Initial growth rates were about fivefold higher in β-chitin than in α-chitin (Fig. 1A). Growth rates in α-chitin did increase at day 7 but were still lower (about twofold) than growth rates in β-chitin. Final cell yields were 5.5-fold higher in β-chitin than in α-chitin. Chitinase activity was over sixfold higher in β-chitin than in α-chitin for 5 days (after the initial lag phase); the difference then decreased to about fourfold (Fig 1B). The initial chitinase activity per cell was eightfold higher in β-chitin than in α-chitin, but after 5 days, no significant difference was observed. As predicted by the higher chitinase activity, preliminary experiments indicate that β-chitin was degraded nearly ninefold more than α-chitin after 14 days. The faster degradation of β-chitin is the result of an increased number of cells, since chitinase activity per cell is similar whether grown in α- or β-chitin after 5 days.

Chitin-degrading proteins excreted by *V. harveyi* following induction with different chitins were collected and analyzed by glycol chitin-SDS-PAGE (Fig. 2). Classification as a chitinase required a distinct band, which depended on the relative intensity of the background for each sample. Bands differing by less than 2 kDa were grouped together. Table 1 summarizes what we found to be the clearest bands. We cite here molecular sizes to identify different chitinases, but these sizes are not accurate (see below).

Cells grown on snow crab and squid pen chitin excreted many of the same chitinases, but they also produced some that were unique (Table 1). Cells grown on snow crab chitin excreted a chitinase migrating around 130 kDa, while those grown on squid pen chitin produced two other chitinases (47 kDa and 125 kDa), which were unique. The chitinases excreted during growth on other chitins were also examined. Several chitin-degrading proteins were present with all chitins, but others appeared unique to a particular type of chitin (Fig. 2). Chitinases of apparent sizes of 42, 77 (possibly more than one chitinase of about the same size), and 91 kDa were present in all treatments. A large-molecular-size chitinase, migrating at about 130 kDa, was excreted with snow crab, colloidal, and regenerated chitins. A 98-kDa protein was excreted with squid,

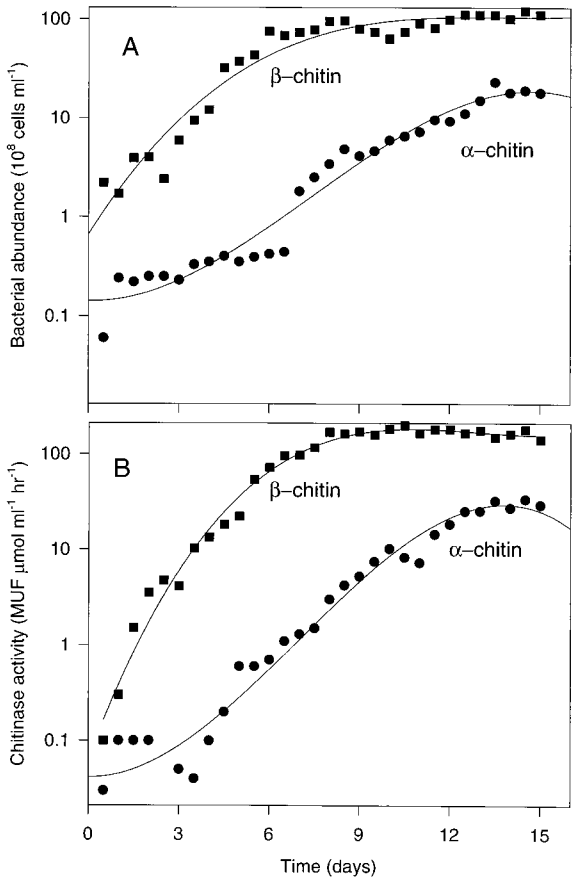

FIG. 1. Growth and chitinase activity of *V. harveyi* grown on structurally different chitins. Cell numbers (A) and chitinase activity (hydrolysis of MUF-diNAG, which released the fluorescent compound MUF) (B) for cells grown on α-chitin (●) or β-chitin (■) are shown.

snow crab, and colloidal chitins, and cells grown on squid and glycol chitin both excreted a 47-kDa chitinase. Unique chitinases were present in extracellular extracts from cells exposed to squid (125 kDa) and glycol (45, 67, and 107 kDa) chitin.

Although equal amounts of protein were loaded, there did not appear to be an equal amount of degradation per sample in the glycol chitin gels. Proteins collected after exposure to glycol chitin resulted in large intense bands, while those collected from cells exposed to regenerated chitin were very faint. These results suggest that not only were different chitinases excreted, but the effectiveness of the different chitinases to a particular chitin depended on the type of chitin the bacteria had been grown on.

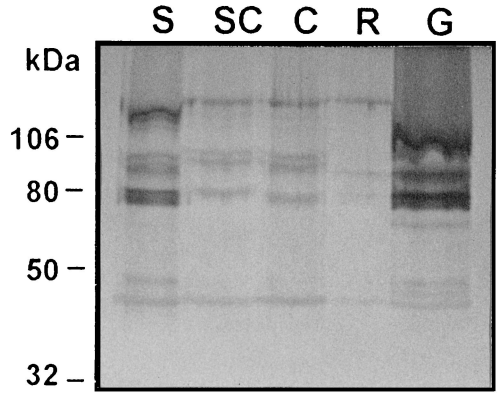

FIG. 2. Chitinases excreted by *V. harveyi* grown on different types of chitin. Lanes: S, squid chitin; SC, snow crab chitin; C, colloidal chitin; R, regenerated chitin; G, glycol chitin. Equal amounts of protein were separated by SDS-PAGE, with glycol chitin added directly to the gel. The bands indicate degradation of the glycol chitin.

TABLE 1. Chitinases excreted in response to growth on different chitins

Molecular mass (kDa) of chitinase	Response to growth on chitin[a]				
	Squid	Snow crab	Colloidal	Regenerated	Glycol
42	+	+	+	+	+
45	0	0	0	0	+
47	+	0	0	0	+
67	0	0	0	0	+
77	+	+	+	+	+
91	+	+	+	+	+
98	+	+	+	0	0
107	0	0	0	0	+
125	+	0	0	0	0
130	0	+	+	+	0

[a] Activities are from Fig. 2. Symbols: 0, no activity; +, activity or hydrolysis detected.

Identification of chitinase genes in *V. harveyi*. The glycol chitin gels suggested that *V. harveyi* BB7 can excrete up to 10 chitinases when grown on different types of chitin. To begin to determine how many of these are original gene products or the result of proteolytic processing, two genomic libraries were constructed and screened to identify chitinase genes other than *chiA*, which was found previously (21). In the plasmid library, the antichitinase antibody recognized a single clone (pPV2) harboring a 7-kb insert which had chitinase activity, as determined with the chitin analog MUF-diNAG and confirmed by other assays (see below). This chitinase gene was named *chiB*. A plaque library was screened for chitinase activity with MUF-diNAG. Seven positive clones were identified and converted to plasmids. Restriction mapping using several enzymes (α*Taq*I, *Eco*RI-*Hin*dIII, *Eco*RI-*Xba*I, *Hha*I, and *Rsa*I) revealed that four of these chitinases were unique; data for α*Taq*I are given in Fig. 3.

In addition to restriction mapping, several lines of evidence indicate that these chitinases are different. Each chitinase hydrolyzed chitin and various analogs of chitin differently. All chitinases were able to degrade chitin in glycol chitin-SDS-PAGE gels, although with varying efficiencies. ChiA was the most vigorous, whereas activities of chitinases C, D, E, and F were barely detectable in glycol chitin gels (Fig. 4). The chitinases were also able to hydrolyze MUF-diNAG after SDS-PAGE (data not shown). ChiC had the greatest activity, while chitinases D, E, and F had the lowest levels of hydrolysis in the MUF-diNAG zymogram. ChiA was the only chitinase able to hydrolyze all of the tested substrates, although hydrolysis of the two chitin analogs was lower than that observed with ChiC (Table 2). ChiA and ChiB were the only chitinases that resulted in measurable degradation of ^{3}H-chitin. In the liquid assay, ChiB also hydrolyzed MUF-diNAG more so than any other cloned chitinase except ChiC, but ChiB could not hydrolyze the chitin analog NP-diNAG. While all the clones were able to hydrolyze MUF-diNAG, only ChiC was able to hydrolyze MUF-NAG. MUF-triNAG was hydrolyzed by ChiA, -B, and -C, with the greatest activity from ChiC. ChiC had low activity on glycol chitin but was the most active in hydrolyzing

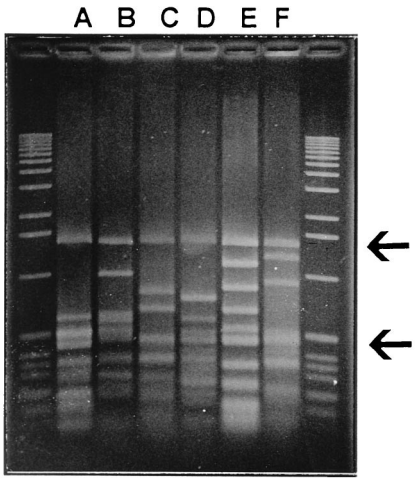

FIG. 3. Agarose gel electrophoresis of plasmid DNA from cloned chitinases digested with α*Taq*I. Lanes: A, clone containing half of *chiA*; B, clone expressing ChiB; C, clone expressing ChiC; D, clone expressing ChiD; E, clone expressing ChiE; F, clone expressing ChiF. Vector bands are designated with arrows. Outside lanes are molecular mass markers (kilobase ladder; Bethesda Research Laboratories).

the chitin analogs. No activity could be detected for chitinases D, E, and F when tested with ^{3}H-chitin or NP-diNAG (Table 2).

Another indication that these chitinases differed is that mobilities in the two zymograms varied. However, it was not possible to measure the apparent molecular weights of these proteins because they were not heat denatured for these zymograms. Anomalous migrations have been observed with proteases in gels containing gelatin (6). In the glycol chitin gel (Fig. 4), ChiA′ was able to degrade the glycol chitin during electrophoresis, resulting in a smear when the amount of protein analyzed was the same as that for the other clones; reducing the amount of ChiA′ gave a single band at 78 kDa. In the Western blot (immunoblot), in which the protein extracts were heat denatured prior to electrophoresis, ChiA′ was 66 kDa (see below). In the glycol chitin gel, larger molecular sizes were

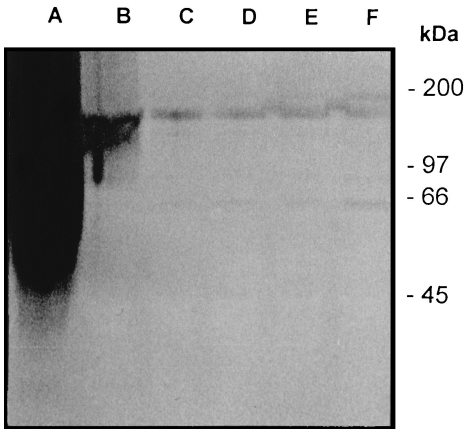

FIG. 4. Chitinase activity of cloned chitinases. Protein extracts were separated by SDS-PAGE, with glycol chitin added directly to gel. Lanes: A, clone expressing ChiA′; B, clone expressing ChiB; C, clone expressing ChiC; D, clone expressing ChiD; E, clone expressing ChiE; F, clone expressing ChiF. Equal amounts of protein were loaded, except for ChiA′, which was loaded at 1/10 the amount analyzed for the others. The dark smear (ChiA′) or band (other chitinases) indicates degradation of the glycol chitin.

TABLE 2. Hydrolysis of various chitin substrates by cloned chitinases and recognition by the antibody against ChiA (anti-ChiA)

Chitinase	Anti-ChiA	Relative activity[a] on:			
		Glycol chitin	^{3}H-chitin	Chitin analog	
				MUF-diNAG	NP-diNAG
A	+++	+++	+	++	++
B	0	++	+	++	0
C	0	+	0	+++	+++
D	0	+	0	+	0
E	0	+	0	+	0
F	0	+	0	+	0

[a] Relative activities: 0, no activity detected above background; +, low level of hydrolysis (2- to 10-fold above background); ++, high level of hydrolysis (10- to 100-fold above background); +++, highest level of hydrolysis (>100-fold above background).

also observed with chitinases B, C, D, E, and F (all over 100 kDa) than in MUF-diNAG gels, where ChiB migrated to 66 kDa and ChiC migrated to 73 kDa. Although these zymograms cannot give accurate estimates of molecular size, the different mobilities of these chitinases confirm that they are different.

The antibody (antichitinase) recognized a 66-kDa protein produced by *E. coli* bearing the plasmid pChiA1 (Fig. 5), which is close to the molecular size (63 kDa) predicted by the sequence data for ChiA′ (21). The chitinase antibody does not cross-react with any other chitinase (Fig. 5). A clone from the plasmid library (pPV2) produced a large-molecular-size protein (122 kDa) which was recognized by the antibody (Fig. 5). The recognized protein did not have chitinase activity (Fig. 4).

DISCUSSION

Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitins can vary by the arrangement of *N*-acetylglucosamine strands, degree of deacetylation, and presence of cross-linked structural components, such as proteins and glucans (3). We had expected some chitinases to be specialized for hydrolysis of particular types of chitin and others to be involved in degradation of all chitins. Indeed, we found that the marine bacterium *V. harveyi* excretes about 10 chitinases when grown on chitin and that the composition of the excreted chitinases varies when cells are exposed to differ-

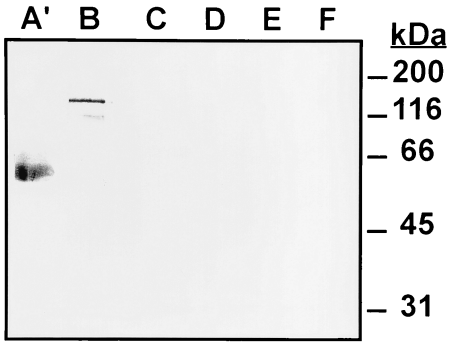

FIG. 5. Western blot of cloned chitinases immunoprobed with anti-ChiA. Lanes: A′, clone expressing ChiA′; B, clone expressing ChiB; C, clone expressing ChiC; D, clone expressing ChiD; E, clone expressing ChiE; F, clone expressing ChiF. Equal amounts of protein were loaded, except for ChiA′, which was loaded at 1/10 the amount analyzed for the others.

ent chitins; some of the proteins are always present, while others are excreted only with particular chitins.

One factor affecting chitin degradation is the arrangement of the *N*-acetylglucosamine strands. The antiparallel α configuration is the most tightly packed and the most commonly found structure of chitin in organisms (3). We observed faster growth by *V. harveyi* on β-chitin than on α-chitin, and as a result, β-chitin seems to be degraded faster, probably because the parallel arrangement of strands in β-chitin permits easier access to bacterial enzymes. Perhaps not surprisingly, cells grown on α- and β-chitin excreted different chitinases. It is perhaps more surprising that the composition of excreted chitinases also varied when the cells were grown on colloidal, regenerated, and glycol chitins, even though all originated from α-chitin. These chitins still vary, however, in strand opening, degree of deacetylation, solubility, and probably the presence of covalently linked components other than *N*-acetylglucosamine.

The end products of chitin hydrolysis, e.g., *N*-acetylglucosamine, glucosamine, and chitobiose, are known to induce chitinase synthesis in *V. harveyi* (12), but these end products should be the same for different chitins and thus would not contain sufficient information for a bacterium to sense the chitin structure. Other factors that induce synthesis of different chitinases in response to different chitins must be involved. Perhaps a cell can determine directly the physical structure of chitin, such as strand arrangement. One example of this mechanism comes from work on *Streptomyces olivaceoviridis*. This bacterium produces a lectin-like protein that binds specifically to crystalline α-chitin (18). A cell could also determine the degree of deacetylation from the relative amounts of glucosamine and *N*-acetylglucosamine released during chitin degradation. The presence of some components covalently linked with particular chitins, such as proteins, could be recognized directly by outer membrane sensory proteins, or cells may respond to by-products released during degradation of the nonchitin components. In addition to separate proteolytic enzymes, bacteria may produce chitinases that contain an additional enzymatic domain, such as a protease. This is the case for *Streptomyces olivaceoviridis*, which produces a 92-kDa protein that can be cleaved into a 70-kDa chitinase and a 22-kDa proteinase (14). In short, efficient utilization of chitin probably requires several enzymes besides chitinases.

To determine the number of chitinase genes and to provide a basis for further work on characterizing chitin-degrading proteins, we screened two genomic libraries for chitinase genes in *V. harveyi*. We found six separate chitinase genes. The chitinases produced by our six clones appear to be unique based on restriction analysis, immunological data, and enzymatic properties. Assuming we have identified all chitinase genes, at least one of the six genes apparently codes for a chitinase that is cleaved or degraded to produce additional chitinases, resulting in the observed total of 10 excreted chitinases. Proteolytic processing of a chitinase to produce additional chitinases is well known (17, 23, 24).

Based on their enzymatic properties, some of these proteins can be tentatively placed into the known classes of chitinolytic enzymes. ChiA and ChiB are most likely endochitinases, which randomly cleave in the center of chitin and produce soluble oligosaccharides (13); these two enzymes were most active on actual chitin substrates (glycol chitin and ^{3}H-chitin), and they could hydrolyze MUF-triNAG. ChiC is probably an exochitinase (or exo-*N*-acetyl-β-glucosaminidase), which cleaves at nonreducing termini (13), since it hydrolyzed the chitin analogs MUF-diNAG and NP-diNAG as well as MUF-NAG and MUF-triNAG most effectively. Finally, since only low activities were detected with ChiD, -E, and -F, identification is more difficult. Our data suggest that these proteins are either chitodextrinases which catalyze hydrolysis of internal glycosidic bonds in soluble oligosaccharides (1) or chitobiosidases (not to be confused with chitobiases which are *N*-acetyl-β-glucosaminidases). These enzymes release chitobiose from the nonreducing ends of the chitin chain (4) and hydrolyze MUF-diNAG.

The great variety of chitin structures which occurs in nature appears to necessitate that bacteria have a large number of different chitinases and other chitin-degrading enzymes. *V. harveyi*, and most likely other bacteria, produce different chitinases in response to the form of chitin they encounter, implying that they can distinguish among different types of chitin. Additional work with these cloned chitinases, and other proteins (such as chitin-binding proteins), is necessary to understand the regulation of chitin degradation by *V. harveyi* and other bacteria in the presence of different forms and structures of chitin.

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